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ABSTRACT

HEYDRICK, FRED P. (Fort Detrick, Frederick, Md.), RALPH F. WACHTER, AND HENRY J. HEARN, JR. Host influence on the characteristics of Venezuelan equine encephalomyelitis virus. J. Bacteriol. 91:2343–2348. 1966.—Alterations in plaque size, virulence, and lipid content of Venezuelan equine encephalomyelitis (VEE) virus were examined for possible interrelationships among these properties during 10 serial passages in embryonated eggs, suckling mice, chick embryo fibroblasts, and L cells. The chick embryo host maintained the same large-plaque and virulence properties of the virus through 10 passages as seen in the original seed. Passage of virus in either L cells or chick fibroblasts rapidly produced populations that were, in the main, intermediate with respect to plaque size and virulence. Passage of virus in suckling mouse brain yielded populations that were intermediate with respect to plaque size only. The nature of the lipid of the virus, in terms of the ratio of petroleum ether-soluble to -insoluble lipid, changed after only one passage in all systems except in chick embryos. Nine additional serial passages failed to enhance these changes in viral lipid, suggesting that the decrease in the large-plaque and virulence properties was not directly associated with changes in lipid content.

The properties of virulence and plaque formation of the equine encephalomyelitis group of viruses have been shown by a number of investigators to be influenced by passage of the virus in various host systems. For example, Marshall et al. (9) found that the large-plaque characteristic of naturally occurring strains of western equine encephalomyelitis virus was maintained consistently during passage in embryonated eggs, less consistently during passage in mouse brain, and was quickly replaced by virus that produced small plaques when passed in chick fibroblast cell culture. The literature contains several reports describing alterations in the properties of Venezuelan equine encephalomyelitis (VEE) virus after passage in cell culture (1, 5, 8, 10, 11). The work of Hardy and Hearn (4), Mussgay and Suarez (11), and Brown (2) indicated a correlation between loss of virulence resulting from passage in cell culture and loss in ability of the virus to form large plaques. In preliminary studies, Hearn and Soper (Bacteriol. Proc., p. 135, 1962) and Heydrick, Cree, and Wachter (Federation Proc. 23:400, 1964) emphasized that different host species varied in their ability to alter plaque size and virulence of VEE virus. An additional factor, the ratio of petroleum ether (PE)-soluble to PE-insoluble viral lipid also appeared to depend upon the host from which the virus was derived (Heydrick et al., Federation Proc. 23:400, 1964). It is the purpose of this paper to report the relationships and changes in plaque size, virulence, and lipid content of viral populations that occurred during 10 serial passages of a single strain of VEE virus, with the use of the chick embryo, suckling mouse brain, chick fibroblasts, and L cells as hosts.

MATERIALS AND METHODS

Virus seeds. The parent egg seed (PES) (5) of the Trinidad strain of VEE virus (10), which had received 13 passages in embryonated eggs, was used to initiate these studies. This seed possessed a titer of $10^{9.2}$ mouse intracerebral (ic) LD_{50} per ml of 20% suspension. Embryo and yolk-sac seeds used to infect various hosts were prepared by inoculating $10^{4.6}$ mouse ic LD_{50} of the PES strain into 10-day embryonated eggs via the allantoic route. After incubation for 24 hr, embryos and yolk sacs were harvested from moribund and dead eggs. These harvested tissues were homogenized separately, and prepared as 20% suspensions in Ringer-Locke solution. After low-speed centrifugation, the supernatant fluid was placed in ampoules and stored at −70 C.

Passage of virus. The embryo and yolk-sac seeds,

which titered $10^{8.5}$ and $10^{8.0}$ mouse ic LD_{50} per ml, respectively, were serially passed 10 times in 11-day embryonated eggs, suckling mice, and in monolayers of L cells and chick fibroblasts (CF). Prior to inoculation, cell monolayers for passage of virus were grown for 48 hr in either T-60 flasks or Roux bottles containing lactalbumin hydrolysate medium with 10% calf serum. The former were inoculated with 0.3 ml and the latter with 1.0 ml of a 1:10 dilution of either the embryo or yolk-sac seed. After an adsorption period of 20 min, the inoculum was removed and fresh growth medium was added. The viral suspensions were harvested after 36 hr of incubation at 37 C. Remaining serial passages were made with the use of harvested tissue culture supernatant fluids as inocula, each of which contained approximately $10^{8.0}$ mouse ic LD_{50}. Serial passages in 1- to 2-day-old suckling mice were initiated in a similar manner by intracerebral injection of 0.03 ml of a 1:100 dilution of either the embryo or yolk-sac seed. Infected brains were harvested after 24 hr, pooled, and stored as 10% suspensions in beef heart infusion broth (BHIB). Subsequent suckling mouse brain serial passages were carried out with approximately $10^{8.0}$ mouse ic LD_{50} of the harvested brain suspensions. Passage of virus in chick embryo was begun with 0.25 ml of a 1:100 dilution of either the embryo or yolk-sac virus. Subsequent serial passages were made with approximately $10^{7.0}$ mouse ic LD_{50} of the harvested embryo pools. All harvested material was stored at −70 C until tested.

Virus assay and plaque size determination. Viral assays were performed by injecting 12- to 14-g mice by the intracerebral (ic) and intraperitoneal (ip) routes with 10-fold dilutions of the preparation in BHIB. Viral titers were expressed as the mouse ic LD_{50} and ip LD_{50} per ml. The CF monolayer plaque technique for intact virus, as described by Colón and Idoine (3), was used to determine plaque size. Plaque diameters were measured to the nearest 0.25 mm. Counts and size determinations were made 2 and 3 days after inoculation; the longer incubation period, however, proved to give more accurate size differentiation. Three groups of plaque sizes were arbitrarily defined: large plaques (4 to 6 mm in diameter), intermediate plaques (2.0 to 3.5 mm), and small plaques (0.5 to 1.5 mm). Two dilutions of inoculum were employed in duplicate to provide between 20 and 120 plaques per plate. Fifth- and tenth-passage preparations were tested simultaneously to minimize variation in plaque size resulting from influences other than those exerted by the host from which the virus was obtained.

Purification of virus. The VEE virus from 20% chick embryo suspension, 10% suckling mouse brain suspension, or infected tissue culture fluid was purified by treatment with Celite, clarification with protamine sulfate, and concentration of the virus by high-speed centrifugation (59,000 × *g*) of the supernatant fluid. The concentrated virus was placed on a continuous sucrose gradient, and centrifuged at 53,500 × *g* for 3 hr in a Spinco SW-25 swinging bucket rotor. The visible virus band, located at a density of 1.14, was removed by puncturing the side of the tube.

Dialysis and lyophilization. Purified virus suspensions from a series of identical gradients were prepared for analysis by diluting the virus band of high sucrose content with 5% sucrose, and sedimenting the virus by high-speed centrifugation (105,000 × *g* for 2 hr). The resuspended virus pellet was dialyzed against distilled water, and lyophilized in small bottles. Samples were further dried to constant weight over phosphorus pentoxide before extraction of lipids.

Lipid determination. Total lipid was determined by direct weighing of the extracted lipid. Lipid was removed with a microextraction apparatus similar to that described by Schaffer et al. (13), by use of the extraction procedure of Thomas (14).

RESULTS

Viral plaque size during passage. As indicated above, embryo and yolk-sac harvests constituted the starting material for passages carried out in parallel in chick embryos, suckling mice, L cells, and CF cells. With one exception that is discussed below, the embryo and the yolk-sac starting materials produced approximately the same results during passage in the four systems. Therefore, only data obtained with virus that originated from the embryo are presented in the tables.

The extent to which the large-plaque characteristic of VEE virus was maintained during passage in these hosts is shown in Table 1. Results are expressed as the percentage of large, intermediate, or small plaques present after the first, the fifth, and the tenth passages. For 10 passages in the chick embryo, approximately 100% of the total population consisted of large-plaque virus. In contrast, infected suckling mouse brain showed a slight decrease in the percentage of large plaques at the fifth passage, and, by the tenth passage, only 6% of the virus population formed large plaques; the remainder of the population formed plaques of intermediate size.

Passage of VEE virus in the cell culture systems resulted in a much more rapid reduction in the percentage of large-plaque particles. After only two passages (not shown in Table 1) in CF cells, large-plaque virus was already in the minority (40%). As shown in Table 1, by the fifth passage all plaques were intermediate in size; at the tenth passage, 87% of the plaques were of intermediate size, and the remaining 13% were small. Results of viral passage in L cells showed a similar trend, but were not identical to those observed after passage in chick fibroblasts. For example, at the fifth passage, two plaque types were discernible. Of these types, 62% were large, although they were conspicuously confined to 4 mm in size rather than the 5- to 6-mm range typical of other large plaques. The remaining 38% were 2 to 2.5 mm. At the 10th passage, large plaques were no longer observed, and the distribution among intermediate and small plaques was almost identical

TABLE 1. *Host influence on plaque formation by Venezuelan equine encephalomyelitis virus*

Host	Distribution of plaque sizes*								
	1st passage			5th passage			10th passage		
	Large	Intermediate	Small	Large	Intermediate	Small	Large	Intermediate	Small
Chick embryo	100†	0	0	100†	0	0	100†	0	0
Mouse brain	100†	0	0	90	4	6	6	94	0
CF cells	100†	0	0	0	100	0	0	87	13
L cells	100†	0	0	62	38	0	0	83	17

* Large, 4 to 6 mm in diameter; intermediate, 2 to 3.5 mm; small, 0.5 to 1.5 mm.

† Incidence of large plaques ranged from 95 to 100% of total. Other results are also expressed as per cent of total.

to that found with CF cells. It is interesting to note, however, that small plaques found with L-cell preparations were confined to sizes at the lower end of the small-plaque range (i.e., 0.5 to 1.0 mm), but small plaques induced by the CF preparations usually approached 2 mm in size.

One instance occurred in which the serial passage of virus from chick embryo and yolk-sac seeds did not yield comparable results. During the passage of virus from yolk-sac starting material in L cells, there appeared to be a greater tendency to preserve the large-plaque-forming characteristic of the virus than in virus that originated from chick embryo. This was most evident at the fourth passage, when only 5% large-plaque virus was recovered from embryo starting material; with the yolk-sac starting material, however, 60% of the virus still produced large plaques. By the 10th passage, 30% of large-plaque virus still persisted.

These results indicate that the chick embryo host maintained the large-plaque characteristics of the virus through 10 passages, and that passage in either L cells or CF produced a much more rapid change in plaque-forming characteristics than did passage in the suckling mouse brain.

Viral virulence during passage. Because the chick embryo seed virus showed a rapid loss of the large-plaque characteristic upon serial passage in vitro, virus from this source was selected for a study of its virulence in mice. Table 2 shows the results of a comparative study with harvests after 1, 5, and 10 passages in chick embryos, suckling mouse brain, L cells, and CF. As long as the serial passages were performed in chick embryos, there was no evidence of attenuation of the virus for mice, since little difference in lethality was found when this strain was assayed by either the ic or ip route. The $\log_{10}$ difference in the titers obtained by these routes (ic LD_{50} − ip LD_{50}) was 0.5 log at the first passage, 0.3 log at the fifth passage, and 0.6 log at the tenth passage. Passage of the chick embryo seed in suckling mice resulted, at the first passage, in a difference of 0.2 log, which increased to a maximum of 0.9 log at the fifth passage. In cell cultures, CF cells produced virus possessing a difference of 1.3 logs at the fifth passage and 2.0 logs at the tenth passage. The difference for the virus grown in L cells was 1.9 logs at the fifth and 2.4 logs at the tenth passage. These results suggest that partial attenuation of virulent VEE virus, as shown by an increased inability to produce lethality by the ip route, paralleled the inability of the virus to form large plaques.

Thus, virus from chick embryos failed to show any appreciable alteration in virulence for mice after 10 serial passages. Virus from suckling mice showed only a slight decrease in virulence that began at the fifth passage and was repeated at the tenth passage. Virus from CF or L cells showed, by the fifth passage, a significant decrease in virulence that was diminished even more by the tenth passage.

Effect of passage in different host systems on the lipid content of the virus. Purified preparations of VEE virus were obtained after 1, 5, and 10 passages of the virus of chick embryo origin both in L cell and in CF monolayers. Purified virus was obtained also after one passage in the chick embryo and one and five passages in suckling mouse brain. These preparations were dialyzed, lyophilized, and analyzed for PE-soluble and PE-insoluble lipids.

The results of these analyses are presented in Table 3. The total lipid content of purified virus from the L-cell host system did not differ significantly at the 1st, 5th, and 10th passage levels, and was similar to the average value of 24.3% (Wachter and Johnson, Federation Proc. 21:461

TABLE 2. *Relative virulence of Venezuelan equine encephalomyelitis virus after passage in various host systems*

Host	Infectivity titers (log_{10} LD_{50}/ml)								
	1st passage			5th passage			10th passage		
	ic*	ip†	ic − ip‡	ic	ip	ic − ip	ic	ip	ic − ip
Chick embryo	9.3	8.8	0.5	9.0	9.3	0.3	9.7	9.1	0.6
Mouse brain	10.3	10.1	0.2	9.7	8.8	0.9	9.3	8.4	0.9
CF cells	8.2	7.8	0.4	8.8	7.5	1.3	9.3	7.3	2.0
L cells	9.0	9.3	0.3	9.2	7.3	1.9	9.7	7.3	2.4

* Titration in mice by the intracerebral route.
† Titration in mice by the intraperitoneal route.
‡ Difference between titers obtained by intracerebral and intraperitoneal routes.

TABLE 3. *Lipid content of Venezuelan equine encephalomyelitis virus propagated in different host systems*

Source of virus	Passage	Per cent lipid		Total	Ratio of PE-sol to PE-insol
		PE-sol*	PE-insol†		
Chick embryo‡	1	15.2	9.7	24.9	1.6:1
Mouse brain	1	6.7	14.7	21.4	1:2.2
	5	7.5	20.0	27.5	1:2.7
CF cell	1	7.4	12.3	19.7	1:1.7
	5	8.0	14.0	22.0	1:1.8
	10	7.0	14.9	21.9	1:2.1
L cell	1	5.8	17.8	23.6	1:3.1
	5	5.7	18.2	23.9	1:3.2
	10	6.1	16.7	22.8	1:2.8

* PE-sol = petroleum ether-soluble lipid.
† PE-insol = petroleum ether-insoluble lipid.
‡ Values for virus of chick embryo origin represent the average values of five preparations, the total lipid of which ranged from 22.6 to 26.6%.

1962) for VEE virus purified from chick embryo suspensions. However, the ratio of PE-soluble and PE-insoluble (PEs–PEi) lipid was 1.6:1 for VEE virus purified from chick embryo at the first passage, and that propagated in L cells was 1:3.1 at the first passage. This ratio for L-cell virus remained approximately the same at successive passage levels. Similarly, neither the total lipid content nor the PEs–PEi ratio of virus derived from CF monolayers was significantly different beyond the first passage. The total lipid content of VEE virus propagated in CF cells appeared to be only slightly lower than that of virus propagated in L cells, but had an average PEs–PEi ratio of about 1:2 compared with a ratio of 1:3 for the L-cell product. The total lipid content and lipid ratio of virus derived from a single passage in suckling mouse brain were similar to those of the virus from CF cells; the apparently higher total lipid of the fifth passage mouse brain viral preparation can not be considered significant without confirmation by further analyses.

These results indicate that a single passage in any one of the host systems determines the nature of the lipid of VEE virus in terms of the relative proportions of PE-soluble and PE-insoluble lipid, and that the total lipid and lipid ratios do not change significantly during 10 passages in either of two tissue culture host systems. This suggests that the change in the virus from large-plaque to other plaque types, and the change in the virulence characteristic of the virus, apparently are not accompanied

by an obvious change in the lipid composition of the virus.

DISCUSSION

The results of these experiments indicate that the host is highly instrumental in the selection of VEE virus types possessing different plaque-forming capabilities, different levels of virulence, and different ratios of PE-soluble to -insoluble lipid. Evidence that as the large-plaque characteristic was lost the virus showed an increased degree of attentuation for test animals supports the hypothesis that these two properties are closely associated during passage in cell culture. The virus encountered during the serial passages in these experiments represents populations that are clearly intermediate to the virulent, large-plaque virus of the parent strain and the attenuated, small-plaque (0.5 to 1 mm) viral strain described elsewhere (4). A similar display of intermediate plaque variants has recently been reported by Walen (15) for vesicular exanthema virus.

Data presented in the present report suggest, however, that there was a significant exception to the concept of correlation between virulence and plaque size. Tenth-passage suckling mouse brain material contained virus that had lost much of its capacity to produce large plaques, as had the L-cell and chick fibroblast preparations, but, in contrast to the latter two preparations, it demonstrated little evidence of attenuation. This phenomenon has been studied further in vivo, resulting in the disclosure of additional genetic alterations in viral properties that will be presented in a subsequent report.

The early (first passage) replacement of virus containing a majority of PE-soluble lipid with virus possessing a majority of PE-insoluble lipid appeared to take place prior to our detection of any loss of large-plaque-forming and virulence properties. Whether the increase in PE-insoluble viral lipid is a prerequisite for the conversion to populations with a preponderance of small-plaque, attenuated viral particles is not known. The data do not fully preclude some type of indirect relationship, because chick embryo virus, which showed little alteration in plaque size or virulence, showed the highest proportion of PE-soluble lipid, but the L-cell preparation, which eventually showed the highest degree of attenuation, showed the highest proportion of PE-insoluble lipid. Since some lipid-containing viruses apparently take on the lipids of their host cells (6, 7, 12, 16), perhaps it is reasonable to postulate that the host cell directly determines the lipid content and lipid ratios of VEE virus independent of the proportion of (genetic) variants in the virus population. It is not surprising, then, that the plaque-forming and virulence properties of virus were more closely related to each other than either is to the factor responsible for the PE-insoluble viral lipid.

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